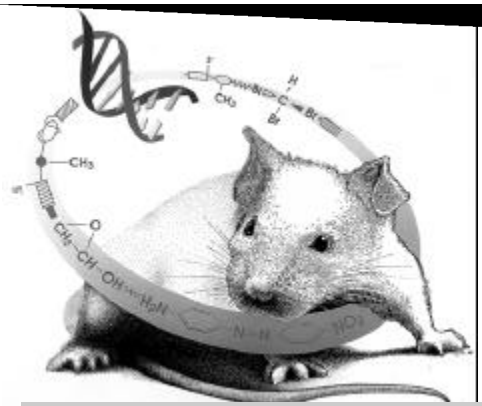

The Tg.AC Workgroup Newsletter

The Tg.AC Workgroup Newsletter is published by The Department of Toxicology and Safety Assessment, Boehringer Ingelheim Pharmaceuticals, Inc. as a means of communication for the HESI's Alternative to Carcinogenicity Testing Committee.

Letter and article submissions are welcome. Persons interested in contributing to the newsletter should contact:

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On the Inside:

March 18, 1998 Meeting: Tg.AC Testing from NTP and Taconic Colony Hemizygotes vs Homozygotes

On March 18, 1998, a meeting was held at the FDA Woodmont facility. This meeting was called by the Tg. AC Workgroup Chairperson in order to; 1) have the FDA, NIEHS, Dupont and Taconic come to an agreement that a problem (presence of a non-responder phenotype) exists with the Tg.AC mouse, 2) determine a means by which to correct the breeding colony(s) of the Tg.AC mice and institute a quality genotyping control system and, 3) determine studies to be done in order to further understand the Tg.AC non-responder phenotype mechanistically. Attendees included members from Boehringer Ingelheim Pharmaceuticals, Inc. (BIPI), the FDA, Taconic, NIEHS, Dupont and Haskell Labs. A presentation was given by Kerry Blanchard of BIPI which described variability in the phenotypic papilloma response in hemizygous and homozygous Tg.AC mice administered PMA by dermal application. Results of a Southern blotting technique developed by Frank Sistare at the FDA were then described (see following article). Sam Phelan from Taconic followed with a discussion on the history of the Tg.AC colony as well as current management and quality control of the Tg.AC line. Ron Cannon then proposed a mechanism by which NIEHS is enriching their NTP Tg. AC homozygous mouse colony to consist exclusively of responders and guard against further genetic instability. This technique included Dr. Sistare's Southern blotting technique as well as phenotyping by PMA (phorbol 12-myristate 13-acetate) treatment and monitoring papillomagenesis. Male and female homozygous Tg.AC mice will be genotyped (using the Southern blot technique) and phenotyped (using PMA treatment). The responders will be bred as well as out-crossed to FVB/N male and female mice. The progeny will again be phenotyped and genotyped. The group agreed that this would be a reasonable means by which to enrich the colony. The Southern blot technique has been transferred to Taconic in order for them to enrich their colony. The technique was determined to be 100% predictive based on analysis performed by NIEHS and the FDA on double-blinded tail samples sent from BIPI. It was estimated that it would take approximately 2 months to correct the colony using the Southern blotting technique described by Dr. Sistare and the breeding method described by Dr. Cannon. A brief discussion on mechanistic studies concluded the meeting.

Research on the Identification of the Non-Responder Phenotype

by Frank Sistare, FDA

Papillomagenesis data from two studies using male and female hemizygous Tg.AC mice were presented at a Meeting on March 18, 1998. Overall, greater than 80% of animals dosed with 1.25 µg of PMA (phorbol 12-myristate 13-acetate) twice per week for 26 weeks, whether in ethanol or in acetone, did not develop papillomas; those that did, developed 2-18 papillomas. Ten of 18 animals developed one or no papillomas with 6.25 µg PMA twice per week in ethanol, and 12 of 18 mice developed one or no papillomas with 6.25 µg twice per week in acetone (these 22 animals are labeled as NON-RESPONDERS). On the other hand, 30% of the animals developed a full papilloma burden, i.e., greater than 32 papillomas per animal (these 11 mice are labeled as RESPONDERS). The copy number was estimated to be approximately 40 using Southern blot analyses of BamH1 restricted and zetaglobin probed genomic DNA taken from tail clips of hemizygous Tg.AC mice, using the endogenous zetaglobin gene as a reference. In comparison to the 3-10 copies originally reported by Leder, this suggests gene amplification has occurred since the late 1980's through successive generations. A unique 2 kb band is apparent only in genomic DNA from RESPONDER mice on Southern blots using tail clip genomic DNA restricted with BamH1 and probed with zetaglobin. Similarly a unique 2.2 kb band is apparent only in RESPONDER mice on Southern blots using DNA restricted with Kpn1. There is no unique band associated with RESPONDER status using Pst1. The robustness of the BamH1/zetaglobin probed Southern approach for predicting RESPONDER was discussed. 27 tail clip samples taken from FDA studies using hemizygous mice dosed with 6.25 µg PMA twice per week were analyzed and 9 of 9 RESPONDERS showed the presence of the 2 kb band on the Southern blot; 0 of 18 NON-RESPONDERS showed the 2 kb band. Double-blinded tail clip samples from Boehringer Ingelheim were similarly analyzed. 23 of 23 RESPONDERS showed the 2 kb band, while 0 of 16 NON-RESPONDERS showed the presence of the 2 kb band. Two models have been proposed which could account for the observed data. In one model the transgene may have integrated into at least two different sites in the genome of the original FVB/N founder mouse, but through successive generations, one of the integration sites was lost in NON-RESPONDERS. The second model, which is favored, but which remains unproven, postulates the formation during the creation of the original transgenic mouse, of a head-to-head orientation of two tandem copies of the transgene. Such an orientation would create an inverted repeat palindrome. This palindrome could form a cruciform structure which could explain the apparent amplification, the propensity for excision/deletion and loss of the responder phenotype, and also could account in a mechanistic sense for the responsive capability of transgene expression. The Southern blot protocol is a 5-6 day procedure. Efforts are currently underway to 1) improve upon the length of time required, 2) improve upon the ability to resolve examples of NON-RESPONDER mice which show only small deletions in the 2 kb band (e.g. 1.9 kb) and, 3) clone and sequence the 2 kb band in order to prove the existence of the proposed palindromic inverted repeat orientation in the diagnostic 2 kb band. These data have been submitted to Toxicologic Pathology for publication.

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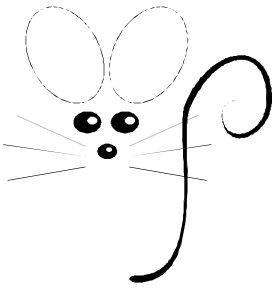
Study Currently Being Conducted at Bristol-Myers Squibb Co.

The Department of Drug Safety Evaluation at Bristol-Myers Squibb's Pharmaceutical Research Institute, Syracuse, New York, is investigating the carcinogenic potential of cyclosporin A when administered orally for 6 months, by diet admixture, to FVB/Tg.AC homozygous transgenic mice as part of the collaborative research program being coordinated by HESI's Alternatives to Carcinogenicity Testing Committee. Three groups of 15 male and 15 female Tg. AC mice are being given cyclosporin A at doses of 7.5, 15, or 30 mg/kg/day in ground rodent diet. As a positive control, a group of mice is being given 100 mg/kg/day of dimethylvinyl chloride (1-chloro-2-methylpropene) in corn oil by gavage. In addition, two groups of mice are receiving either unadulterated rodent chow or corn oil by gavage as the diet and gavage negative controls, respectively. Each mouse is being observed daily for viability, examined twice weekly for detection of external or subcutaneous tissue masses, and observed every 2 weeks for clinical signs of toxicity. At the end of the 26-week dosing period, the mice will be subjected to a complete histopathological analysis and blood will be obtained for verification of exposure. We are currently in the fourth week of the study. To date, no skin changes or drug-related adverse effects have been observed. Final results are expected to be reported in July 1999.



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Frequency of Non-Responsive Phenotypes in Hemizygous and Homozygous Tg.AC Mice

by Kerry T. Blanchard, BIPI

In the first issue of the Tg.AC, "Identification of a Non-Responsive (NR) Phenotype" was described by Drs. Tennant and Stoll. Since this time, questions arose as to the frequency of this non-responsive phenotype in the mouse colonies especially for interpretation of past studies as well as ongoing studies.

Boehringer Ingelheim Pharmaceuticals, Inc (BIPI) has a historic database of 13 hemizygous positive control groups treated with TPA. These groups are presented in chronological order (from studies beginning in 1995 up to the present) in Figure 1. The initial three experiments, conducted in 1995-96, yielded positive control data that was consistent with data generated at the NIEHS laboratory (males, 70-90% responding; females, 40-70% responding). The results of the fourth experiment, in which only 1/10 (10%) males and 3/10 (30%) females responded positively to TPA, were unexpected. In the two most recent studied (numbers 12 & 13, Fig. 1), the responsiveness of hemizygous animals is nearly non-existent (1 of 60 animals treated with TPA developed papillomas). Furthermore, it is our experience that "responding" animals routinely generate greater than 20 papillomas when administered TPA; however, the one animal that responded in experiment 12 developed only 2 papillomas. These results highlight the necessity of not only randomizing animals amongst groups, but also including a positive control group in every study. Only then can a meaningful interpretation of the data be completed **especially** when the Test Article on study is judged to show no evidence of carcinogenicity.

We have also administered TPA to homozygous Tg.AC mice in our laboratories. The results of 8 homozygous positive control groups (conducted between 1996 and the present) are shown in Figure

2. On average approximately 75% of homozygous animals respond (range: 40% to 100%). The responsiveness of the homozygote was certainly more predictable than that of the hemizygote. Although responsiveness in the positive control groups is usually less than 100%, the frequency of homozygous responders was significant allowing for a clear interpretation of the results. However, we should not lose sight of the hemizygous results and the loss of responsiveness in the most recent studies (Fig. 1). A positive control is essential for every study.

Fig. 1: Hemizygous TPA Positive Controls

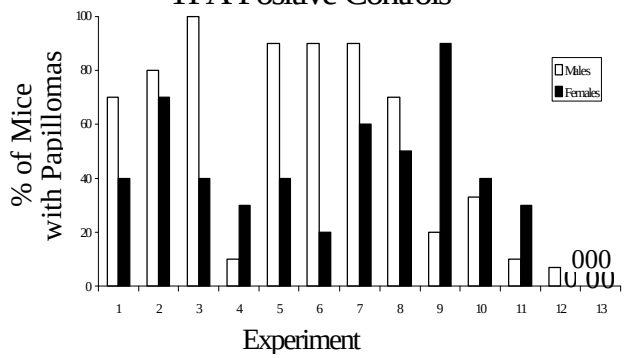
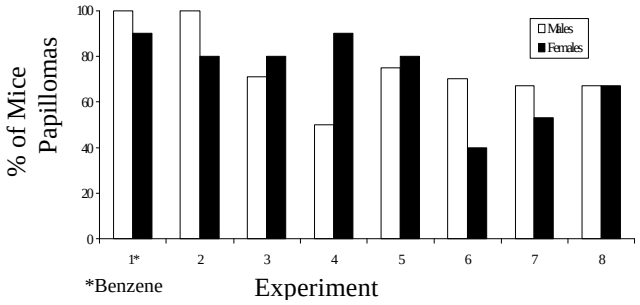


Fig. 2: Homozygous TPA Positive Controls



These results have been submitted for publication:
K.T. Blanchard, *et al.* (1998) Dermal Carcinogenicity in Transgenic Mice: Relative Responsiveness of Male and Female Hemizygous and Homozygous Tg.AC Mice to 12-O-Tetradecanoylphorbol 13-Acetate (TPA) and Benzene, *Toxicologic Pathology*, submitted

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Articles of Interest

Goelz, M.F., Mahler, J., Harry, J., Myers, P., Clark, J., Thigpen, J.E., and Forsythe, D.B. (1998) Neuropathologic findings associated with seizures in FVB mice. Lab Animal Sci 48(1): 34-37.

Wright, J., Hansen, L., Mahler, J., Szczesniak, C., and Spalding, J. (1995) Odontogenic tumors in the v-Ha-ras (Tg.AC) transgenic mouse. Archives of Oral Biology 40: 631-638.

Hansen, L.A., Trempus, C.S., Mahler, J.F., and Tennant, R.W. (1996) Association of tumor development with increased cellular proliferation and transgene overexpression, but not c-Ha-ras mutations, in v-Ha-ras transgenic Tg.AC mice. Carcinogenesis 17(9): 1825-1833.

NTP Update

by William Eastin, NTP, NIEHS

- The NIEHS/NTP website on the transgenic studies has been modified. This site can be found at http://ntp-server.niehs.nih.gov/Main_Pages/transgen/Transgen_default.html. New to this site is the addition of the NTP historical control pathology tables and body weight gain and survival curves.

- The results of completed studies with Tg.AC (and $p53^{def}$) mice were presented to the NTP's Board of Scientific Counselors February 5, 1998. The abstract for the NTP studies presented at that meeting follows:

"National Institute of Environmental Health Sciences researchers are exploring the utility of transgenic mice to study mechanisms of carcinogenesis. Two of these mouse models the Tg.AC (carrier of an activated mouse H*ras* oncogene) and the p53+/- (hemizygous for the tumor suppressor gene) have genetic alterations that appear to hasten their expression of chemically induced tumors. These two models have been proposed as a basis for new strategies for identifying chemical carcinogens and assessing risk. The National Toxicology Program (NTP) is conducting a series of studies with these two transgenic strains to further examine their strengths and weaknesses for identification of documented rodent and human carcinogens.

In this first evaluation, candidates for study were drawn from the NTP historical database of 2-year rodent carcinogenicity studies and the open literature (primarily for drugs). Results with this first set of 11 chemicals tested in transgenic mice compared with previous findings with the traditional 2-year rodent assays and literature on human tumor findings appear to support the premise advanced by Tennant, et al. (1) that these models have the potential to serve as more rapid and less expensive test systems to identify carcinogens."

[1] Tennant, R.W.,French, J.E. and Spalding, J.W. Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models, Environ. Health Perspect., 103, 942-950 (1995).

- New studies are currently being conducted at NTP contract testing laboratories in homozygous Tg.AC by the topical and oral routes to determine the carcinogenic potential of WY 14643, diethylhexylphthalate, melphalan, cyclophosphamide, diethylstilbestrol and 17a-ethinyl estradiol.

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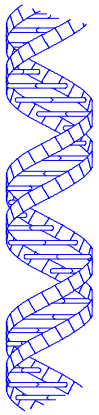
Upcoming Meetings and Events

☺ 8th International Congress of Toxicology
Paris, France

July 6-11, 1998

☺ Gene Environment Interactions: Emerging Issues, Technologies and Biological Paradigms
Barton Creek Conference Resort, Austin, Texas

December 2-5, 1998



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